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Neutralization effects of interleukin-6 (IL-6) antibodies on sulfur mustard (HD)-induced IL-6 secretion on human epidermal keratinocytes☆

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Abstract

The proinflammatory cytokine human interleukin-6 (hIL-6) plays an important role in the early and late courses of inflammation, trauma, and wound healing caused by sulfur mustard (HD). Previously, we demonstrated that hIL-6 might be involved in the early event of structural changes of the signal transducer glycoprotein, which indirectly initiates the cascade of events, such as skin irritation and blister formation observed in the pathophysiology of HD injury. In this present work, we focus on the neutralization effect of IL-6 antibodies with regard to the modulation of hIL-6 secretion. Levels of secreted cytokine hIL-6 in normal human epidermal keratinocytes (NHEK) stimulated with HD (10^{-4} M) and incubated for 24 h at 37 °C were determined by enzyme immunoassay, protein immunocytologic assay and reverse-transcriptase-polymerase chain reaction (RT-PCR). The ratio of HD-treated NHEK to constitutive non-stimulated NHEK controls (S/C) on the induction of hIL-6 is reported. S/C was four-fold higher than non-stimulated NHEK controls as determined by ELISA. By using a more sensitive immunocytologic assay, Luminex^{100TM}, the increment was verified. hIL-6 levels in NHEK stimulated with HD were 21 ± 11 ng/mL as measured by Luminex^{100TM}. The messenger RNA expression of the cytokine (hIL-6) gene was analyzed semiquantitatively. RT-PCR demonstrated that HD induced an increase in the transcription of hIL-6 gene. Selective immunosuppression, using IL-6 neutralizing antibodies, led to a reduction of such expression of HD-induced transcription of hIL-6 in human keratinocytes. The neutralization by pre-incubating NHEK with monoclonal anti-IL6 antibodies decreased hIL-6 secretion by 76% ± 1.8 (*$P < 0.05$).

Keywords: Sulfur mustard; HD; Human interleukin-6; Anti-IL-6 antibodies; Keratinocytes; Human skin cells; Immunocytologic assay; RT-PCR

1. Introduction

Cytokines are important regulators of immune and inflammatory reactions in the skin and may contribute to inflammatory blister induction. Rhodes et al. examined the profiles of human interleukin-6 (hIL-6) in fluid of spontaneous blisters in the immune-based inflammatory disorders bullous pemphigoid, allergic contact dermatitis and toxic epidermal necrolysis. High levels of hIL-6 were reported and found in patients with inflammatory blisters that originated from toxic epidermal necrolysis. Their observations confirm that different secretion-related patterns of hIL-6 occur in a range of blistering disorders. Rhodes et al. (1999) also concluded that individual cytokines have different actions depending on the cytokine microenvironment. Therefore, defining the functional contribution of individual cytokines to skin blistering will require experimental injection of pure, recombinant cytokine into the skin, or the application of specific cytokine antagonists or anti-cytokine antibodies.

We have postulated that hIL-6 is an important regulator of immune and inflammation reactions in the skin caused by sulfur mustard (HD) and may contribute to inflammatory blister induction (Arroyo et al., 2001). In the target tissue, the hIL-6 passes through the plasma membrane by simple

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diffusion and binds to specific receptor proteins in the nucleus. The glycoprotein-receptor complexes act by binding to highly specific DNA sequences and altering gene expression. This glycoprotein binding triggers changes in the conformation of the receptor proteins so that they become capable of interacting with specific transcription factors. The bound IL-6-receptor complex can either enhance or suppress the expression (transcription into messenger RNA) of specific genes adjacent to the cytokine response elements (CREs), and thus the synthesis of the gene protein products.

Human IL-6 has two disulfide bond links (Rock et al., 1994) and results from mutagenesis experiments have revealed that only the second disulfide bridge (Cys_{74}–Cys_{84}) is essential for IL-6 bioactivity and receptor binding (Rock et al., 1994). The alkylation and cross linking of cysteine-IL-6 glycoproteins by HD may contribute to its toxicity. It is clear that proteins can be cross-linked by HD (Byrne et al., 1996); whether the crosslinking of IL-6 glycoprotein contributes to HD toxicity remains to be established. Thus, if one supplies cysteine, the growth factor hormones involved in cell proliferation and cell growth are not susceptible to HD injury (Sidell et al., 1997). Other investigators have shown that L-oxothiazolidine-4-carboxylate (OTC, an intracellular up regulator for cysteine) and *N*-acetyl-L-cysteine (NAC) appeared to have partial efficacy against HD, OTC being more effective than NAC (Gross et al., 1997) in human peripheral blood lymphocytes (PBL). From our data, the possibility cannot be excluded that hIL-6 is involved in the early event of structural changes of the signal transducer glycoprotein, which indirectly initiates the cascade of events, such as skin irritation and blister formation observed as described in the pathophysiology of HD injury (Sidell et al., 1997; Arroyo et al., 2001).

Since keratinocyte-derived hIL-6 plays a role in the development of epidermal skin inflammation in HD-stimulated systemic inflammatory reaction, neutralization effects of IL-6 antibodies were investigated. The neutralization effects of anti-IL-6 antibodies in the hIL-6 secretion levels induced by HD on human epidermal keratinocytes are reported. Finally, a discussion in the light of their potential therapeutic value is presented.

2. Materials and methods

2.1. Chemicals

Sulfur mustard (2,2′-dichlorodiethyl sulfide; HD) was acquired from the US Army Soldier Biological Chemical Command (Aberdeen Proving Ground, MD, USA). Five microliters (5 μL) of HD was dissolved in keratinocyte growth media (KGM™, Clonetics®, BioWhittaker, Inc., Walkersville, MD, USA) to a final concentration of 4 mM. Mouse (monoclonal) anti-interleukin-6 antibody (catalog #AHC0863) and goat (polyclonal) anti-IL-6 antibody (catalog #AHC0963) were obtained from Biosource International, Camarillo, CA, USA. Human IL-6 UltraSensitive (US) kit was purchased from Biosource International, Camarillo, CA, USA and was used for the quantitative determination of soluble human IL-6 in cell culture supernatant. All the reagents were of the highest purity available.

2.2. Cell culture and chemical treatments

Cryopreserved, normal adult human epidermal keratinocytes (NHEK) were grown in keratinocyte basal medium (Clonetics®, BioWhittaker, Inc., Walkersville, MD, USA) at 0.15 mM calcium and supplemented with 5 mg/mL insulin, 0.1 ng/L recombinant epidermal growth factor, 0.4% bovine pituitary extract, 0.5 mg/mL hydrocortisone, 50 mg/mL gentamicin and 50 ng/mL amphotericin-B (henceforth referred to as keratinocyte growth medium or KGM™) (Boyce and Ham, 1985). The second passage of keratinocytes were subcultured in 150 cm^2 flasks at a seeding density of $\cong 2.5 \times 10^3$ cells per cm^2 in KGM™. When NHEK reached a desired density of $\sim \geq 80\%$ in 150 cm^2 flasks, the cells were exposed to HD (10^{-4} M) for 24 h at 37 °C as previously reported (Arroyo et al., 2001, 2003).

NHEK in 150 cm^2 culture flasks containing fresh KGM™ media were exposed to 10^{-4} M HD per flask. This concentration of HD (10^{-4} M) was estimated to be needed to produce the observed effects in the skin of HD casualties (Vogt et al., 1984; Papirmeister et al., 1991; Sidell et al., 1997). The culture flasks were maintained at room temperature in a chemical fume hood for approximately an hour to allow venting of volatile HD agent and then transferred to a CO_2 incubator at 37 °C for 24 h. Cell viability experiments (trypan blue exclusion and MTT-assay) of controls (non-stimulated) and HD-stimulated cells showed that the cell viability for controls was greater than 95% of surviving cells and approximately 85% or lower (Guzman et al., 2000) with 10^{-4} M HD under similar culture conditions.

Neutralizing IL-6 antibodies were added 24 h before HD stimulation. No significant differences were observed when the neutralizing antibodies were left in the culture media at the time of HD addition or when the cultures were rinsed between antibody treatments and HD stimulations. For instance, a 64% level of neutralization was determined when the anti-IL-6 antibodies (0.01–0.05 ng/mL) were present in the culture media at the time of HD addition. A 65% level of neutralization was determined at the same concentrations of anti-IL-6 antibodies when the cultures were rinsed between treatments and HD stimulation at the same concentrations of anti-IL-6 antibodies. The quantity of cytokine, hIL-6, in the supernatant was determined using an enzyme-linked immunosorbent assay (ELISA, human IL-6 UltraSensitive kit) and an immunocytologic assay, Luminex^{100TM} (Luminex®, Austin, Texas, USA). Levels of hIL-6 were measured in controls (non-stimulated), HD-stimulated, pretreated antibodies cell controls, and pretreated antibodies followed by HD stimulation cells.

2.3. hIL-6 ELISA quantification

The standard cytokine ELISA was applied as previously described (Arroyo et al., 2001, 2003). ELISA experiments were performed as described in the manufacturer's literature (catalog #KHC0064, Biosource International, Camarillo, CA, USA) and were used for the determination of soluble hIL-6 in cell supernatants. This assay employs a monoclonal antibody specific for hIL-6 that has been pre-coated onto a microplate. Standards and NHEK supernatants are pipetted into the wells and the immobilized antibody is bound to any hIL-6 present. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for hIL-6 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate is added to the wells. After a predetermined incubation period the color development is stopped and the intensity of the color is determined. The developed color is proportional to the amount of hIL-6 bound in the first step.

2.4. Luminex^{100TM} analysis system reagent kits

The general immunocytologic assay protocol is very similar to standard cytokine enzyme-linked immunosorbent assays (Taylor et al., 2001). Briefly, beads, buffers, and samples (including standards of known biomarker content, control specimens and unknown) were pipetted into the wells of a filter-bottom microplate. During the first incubation, the specific biomarker bound to the immobilized (capture) antibody on one site. After washing, a biotinylated antibody specific for a different site on the same biomarker was added. During the second incubation, this antibody bound to the immobilized biomarker captured during the first incubation. After removal of excess second antibody, streptavidin-*R*-phycoerythrin (SAV-RPE), a fluorescent antibody, was added. The SAV-RPE bound to the biotinylated antibody to complete the four-member sandwich. After a third incubation and washing to remove all the unbound dye, the fluorescent antibodies bound to beads were quantified. The intensity of the fluorescence is directly proportional to the concentration of biomarker present in the original specimen.

2.5. RT-PCR experiments

Cultured NHEK were collected and homogenized in a small volume of TRIzol® Reagent (Invitrogen Corp., Carlsbad, CA, USA). The total cellular RNA was extracted according to the manufacturer's instructions described in TRIzol® Reagent protocol (Invitrogen Corp., Carlsbad, CA, USA). We performed the homogenization procedure using ~5 to 10 × 10^6 cells placed in 2.0 mL centrifuge tube. The aqueous solution was transferred to a fresh 1.7 mL tube and precipitated by using isopropyl alcohol (0.5 mL per 1 mL of TRIzol® Reagent). This reaction mixture was incubated for 15 min at room temperature and then centrifuged for 15 min (12,000 × *g*) at 4 °C.

After removing the supernatant the RNA pellet was washed once with 75% ethanol by adding at least 1 mL of ethanol per 1 mL of TRIzol® Reagent. The RNA sample was mixed by vortexing and centrifuged (7500 × *g*) for 5 min at 4 °C. These two steps were repeated for a second wash. The RNA pellets were air-dried for 10 min at room temperature and centrifuged with caps open to remove traces of leftover ethanol, if necessary. The RNA samples were dissolved in RNase-free water (50 μL) by repetitively drawing up and expressing the solution through the pipette tip. The samples were vortexed and placed on ice for 10 min. The reaction was stopped by heating the samples for 10 min at 65 °C, and then the samples were placed on ice for 5 min.

Genomic DNA was removed by adding 0.1 volume 10× DNase reaction buffer, and 1 μL DNase I enzyme and mixing the solution thoroughly followed by a 30-min incubation at 37 °C. After incubating, 1.0 μL of 0.125 M EDTA (Invitrogen Corp., Carlsbad, CA, USA) was added and the reaction mixture was heated for 10 min at 65 °C.

The RNA agarose gel was prepared by using a 1% agarose gel (0.5 g) in a 1 × Na_2HPO_4 buffer. The gel was run at 100 V until migrated ~3/4 down the gel, ~35–40 min. Bands on the gel were visualized by using the GeneGenius Image Analysis system (Syngene Inc., Frederick, MD, USA) and analyzed by the GeneSnap Program (Syngene Inc., Frederick, MD, USA). The conversion of total RNA to cDNA was completed using Ambion®'s protocol (Armored RNA®, Ambion Inc., Austin, TX, USA) for reverse transcriptase of RNA (catalog #1760–1774). The primers that were used are proprietary by Ambion Inc., Austin, TX, USA (catalog #5329).

When the RT-PCR reaction was completed, the PCR products were visualized by UV illumination following electrophoresis through a 1.5% agarose gel (UltraPure, Sigma®, St. Louis, MO, USA) at 80 V for ~35 min using 8 μL of RT-PCR product along with 2 μL 6× or 10× loading dye. The agarose gel was prepared by using 0.75 g of agarose in 50 mL of Tris-borate-EDTA (1× TBE; Fluka, BioChemika, Switzerland) buffer (89 mM boric acid, 2.5 mM EDTA, pH 8.2) stained with 0.5 μg/mL ethidium bromide. Gels were scanned using the GeneGenius Image Analysis system and analyzed using the GeneSnap Program.

2.6. Data analysis

All data points were assayed at least in quintuplicate. Data presented are from single experiments that are representative of other similar experiments. Each experiment resulted in mean values computed from numerous determinations for controls, HD-stimulated, pre-treated, and pre-treated/HD-stimulated samples, and reported in pg or ng of cytokine/mL of cell supernatant. Each of these means had a standard deviation associated with it. Statistical significance was determined by the RS/1 Multicomparison (Bolt, Beranek and Newman, Cambridge, MA, USA) procedure using the Wilkes–Shapiro test for normality and

Dunnett's test for multiple comparisons with a common control group. When variance was nonhomogeneous, multiple comparisons utilizing the Bonferroni adjustment of the Student's t-test were performed (Ryan, 1989). Statistically significant differences were reported when $^{*}P < 0.05$ and $^{**}P < 0.1$.

3. Results

We previously concluded and reported that HD induced hIL-6 secretion in human epidermal keratinocytes (Arroyo et al., 1999, 2001, 2003). The secretion of hIL-6 in NHEK as determined by the ratio of stimulated sample to control sample (S/C) was 3.6 ± 0.5 as assessed by ELISA (data not shown).

The secretion levels of hIL-6 were 5 to 30 ng/mL at an index of cell viability ≅85% as verified by Luminex^{100TM} (Table 1). As illustrated in Table 1, an increase in the secretion hIL-6 levels was evident when NHEK were stimulated with HD (10^{-4} M) for 24 h at 37 °C as determined by Luminex^{100TM}. Levels of hIL-6 secreted by non-stimulated NHEK controls were diversified as a function of cell confluence as shown in Table 1. Furthermore, the secretions of hIL-6 depend on the individual donors and cell confluence (Table 2).

This observation is in agreement with other laboratories that have reported that the secretion of hIL-6 by skin cells varies among volunteers (Kupper et al., 1989).

Pretreatment of NHEK with anti IL-6 mouse or goat antibodies (0.25 μg/mL) for 24 h followed by stimulation with HD (10^{-4} M) for an additional 24 h at 37 °C neutralized the secretion of hIL-6 induced by HD. Fig. 1 shows typical representations of the semiquantitative analyses of these two anti IL-6 antibodies in NHEK. The secreted levels of hIL-6 show a large difference between control values and the two different antibody treatments. These secreted levels of hIL-6 reflected different donors. The histogram of monoclonal antibody represents the secreted levels of a Caucasian female, 25 years old and in good health. The determined secretion for this donor was 1.0 ± 0.4 ng/mL for control and 8.2 ± 0.3 ng/mL for HD-stimulated cells. The histogram of the polyclonal antibody illustrates the secreted levels of hIL-6 by a Black female, 34 years old and in good health. The control levels were 0.3 ± 0.2 and 1.3 ± 0.3 ng/mL for HD-stimulated. Finally, the percentage of neutralization was estimated to be ≅96% for the monoclonal (mouse) antibody and ≅83% for the goat (polyclonal) antibody as determined by the immunocytologic Luminex^{100TM} assay.

To determine the relationship between the dose of the monoclonal antibody hIL-6 and the effect on the hIL-6 release profile observed in the presence of HD, dose-response curves were generated using Luminex^{100TM} assay. We modeled the action of the antibody in reducing HD evoked hIL-6 release as a simple drug-receptor interaction under the assumption that the action of this antibody is mediated through binding to receptors in NHEK. For this simplified system, Eq. (1) describes the relationship between the concentration of the antibody and the observed profile effect (Martin, 1978). The following values were measured: M_c, the secretion of hIL-6 in control cells; $M_{C,HD}$, the secretion of hIL-6 in HD-stimulated cells; $M_{C,A}$, the secretion of endogenous hIL-6 in cells pretreated with the antibody and $M_{C,A,HD}$, the secretion of hIL-6 in HD-stimulated cells previously pretreated with the antibody. Thus, the secretion of hIL-6 due to HD is the difference $M_{C,HD} - M_C$ and the secretion of hIL-6 due to HD in the presence of the antibody is the difference $M_{C,A,HD} - M_{C,A}$. The measured effect of the antibody is defined as the percentage by which the secretion hIL-6 due to HD stimulation is reduced in the presence of the antibody at a given concentration:

$$E = \frac{(M_{C,HD} - M_C) - (M_{C,A,HD} - M_{C,A})}{M_{C,HD} - M_C} \quad (1)$$

To examine this relationship, NHEK cultures were pretreated before HD stimulation with the monoclonal anti IL-6 anti-

Table 1
Human IL-6 detected by Luminex^{100TM} in stimulated NHEK culture with HD (10^{-4} M) for 24 h at 37 °C

Cytokine	NHEK percent confluence	NHEK control (ng/mL)	HD-stimulated 10^{-4} M (ng/mL)	S/C[a]
hIL-6	75	1.0 ± 0.2	4.7 ± 0.2	4.7
hIL-6	89	1.8 ± 0.1	11 ± 0.1	6.1
hIL-6	93	2.4 ± 0.1	30 ± 0.1	12.5

[a] Ratio of stimulated to control NHEK.

Table 2
Human IL-6 detected by Luminex^{100TM} in stimulated NHEK culture with HD (10^{-4} M) for 24 h at 37 °C

Donor information	NHEK percent confluence	NHEK control hIL-6 (ng/mL)	HD-stimulated 10^{-4} M hIL-6 (ng/mL)	S/C[a]
Race: Black sex: male	93	2.4 ± 0.3	27 ± 0.3	11.3
Race: Black sex: female	85	1.3 ± 0.1	13 ± 0.4	10.0
Race: Caucasian sex: female	75	1.1 ± 0.2	4.2 ± 0.2	3.8
Race: Asian sex: male	75	1.3 ± 0.1	4.0 ± 0.2	3.1

[a] Ratio of stimulated to control NHEK.

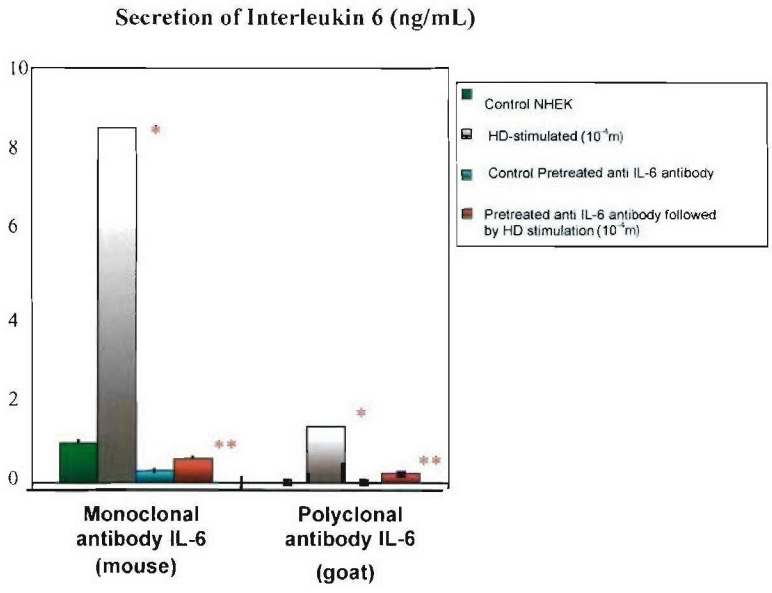

Fig. 1. Typical representations of HD induced hIL-6 release in NHEK neutralized by mouse (monoclonal) and goat (polyclonal) hIL-6 antibodies. NHEK cells were pretreated for 24 h with 0.25 μg/mL of the hIL-6 antibodies, respectively, and then stimulated with HD (10^{-4} M) for 24 h at 37 °C. Supernatants were collected and analyzed by the Luminex^{100TM} assay as described in Section 2. Results are expressed as the mean ng/mL of h IL-6. Neutralization of the release of hIL-6 in NHEK pretreated with mouse (monoclonal) IL-6 antibody is illustrated in the histogram (left side). The neutralization effect for the monoclonal antibody was estimated to be 0.96. The neutralization of the secretion of hIL-6 in cultured NHEK pretreated with the goat (polyclonal) IL-6 antibody is shown in the right side histograms. The neutralization effect was estimated to be 0.84 for the polyclonal antibody. These intervention studies were represented as an average of five independent determinations. Statistical results as follows: $^{*}P < 0.05$ and $^{**}P < 0.1$ vs. NONE by Bonferroni's test.

body. Table 3 shows the quantitative values of the percentage by which HD-induced hIL-6 secretion in NHEK was neutralized by the monoclonal anti IL-6 antibody as measured by Luminex^{100TM}. At the maximum concentration tested, this monoclonal antibody counteracted the response by ~75%. Secretion of hIL-6 induced by HD was neutralized in varying degrees at different concentrations of the antibody. The observed variability in the calculated E could be attributed to different cell confluence for each particular dose-response experiment. Anti-mouse IL-6 antibody appeared to decrease the hIL-6 levels in the HD-stimulated NHEK at low doses 0.01–0.10 μg/mL (Table 3). These decreases were statistically significant, $^{*}P < 0.05$.

The changes in hIL-6 mRNA levels in non-stimulated NHEK were determined as a function of time (Fig. 2A).

Table 3
The percent of E^{a} by which HD-induced hIL-6 in NHEK^{b} was neutralized by the monoclonal antibody as determined by Luminex^{100TM}

Monoclonal antibody IL-6 (μg/mL)	E^{a} (%)	E (S.D.)
0.01	63.8*	4.5
0.02	65.8*	5.5
0.04	65.2*	8.3
0.05	66.1*	8.7
0.06	66.8*	6.5
0.08	67.1*	6.4
0.10	76.4*	4.6

[a] E = effect (reduction) = $[(M_{C,HD} - M_C) - (M_{C,A,HD} - M_{C,A})]/(M_{C,HD} - M_C)$.
[b] Cell density (≅10^6 cells/mL).
* $P < 0.05$.

Because the data showed that about 30-attomol/μg of hIL-6 mRNA were recovered after 24 h of stimulation, the neutralization antibody studies were performed after 24 h. These results were also expressed as a percentage of control values (Fig. 2B). Anti-mouse IL-6 antibody decreased the hIL-6 mRNA levels in the HD-stimulated NHEK in a concentration-dependent manner (Fig. 2C). The decreases were statistically significant at all the concentrations ($^{*}P < 0.05$). Anti-mouse IL-6 antibody (100 μg/mL) appeared to decrease by a factor of 0.56 the levels of hIL-6 mRNA in the HD-stimulated cells (Fig. 2C).

Human IL-6 transcription induced by HD was determined by reverse-transcriptase-polymerase chain reaction (RT-PCR) amplification. NHEK were stimulated with HD (10^{-4} M) and the cytokine hIL-6 transcript was detected by RT-PCR. Fig. 3 illustrates the obtained amplification of mRNA from HD-stimulated cells and anti IL-6 antibodies treated cells. As expected, the constitutive gene for hIL-6 was amplified by PCR on DNA from both HD-stimulated and non-stimulated cells (Fig. 3). RT-PCR demonstrated that HD mediated an increase in the transcription of hIL-6 gene. Our results led us to speculate that HD, in addition to causing DNA mutations and rearrangements, affects cell growth and differentiation by directly changing the structural integrity and binding properties of hIL-6. From our data, the possibility cannot be excluded that hIL-6 might be involved in the early event of structural changes of the signal transducer glycoprotein that indirectly initiates the cascade of events such as skin irritation and blister formation observed in HD pathophysiology. Furthermore, the application

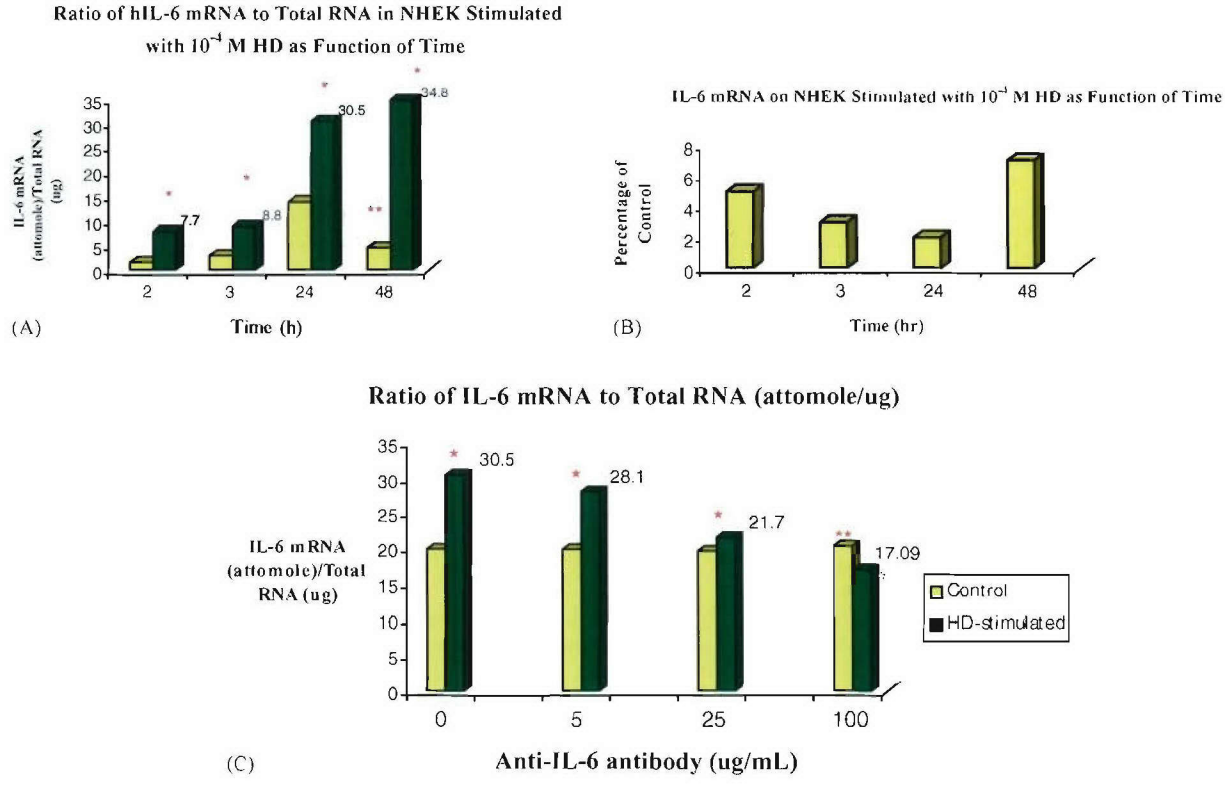

Fig. 2. (A) Ratio of hIL-6 mRNA to total RNA in NHEK stimulated with 10^{-4} M HD as a function of time: 2, 3, 24, and 48 h. The yellow histogram represents the control group, and the HD-stimulated group is illustrated with green histogram. Statistical results: $^{*}P < 0.05$ and $^{**}P < 0.1$ vs. NONE by Bonferroni's test. (B) The results of hIL-6 mRNA as a function of time expressed as a percentage of the control values are given. (C) Effects of anti-IL-6 monoclonal antibody on the hIL-6 mRNA levels in the non-stimulated NHEK controls after HD stimulation. The hIL-6 mRNA expression as assessed by RT-PCR amplification of non-stimulated cells and of cells pretreated with 10, 50, and 100 μg/mL antibody. Statistical results: the difference between concentrations, $^{*}P < 0.05$; the difference between the interaction $^{**}P < 0.1$ vs. NONE by Bonferroni's test.

of IL-6 neutralizing antibodies led to a reduction of such expression of HD-induced transcription of hIL-6 in human keratinocytes. The neutralization by pre-incubation NHEK with monoclonal anti-IL-6 antibodies (i.e., 100 μg/mL) decreased hIL-6 secretion by 43.53% ± 1.75 ($^{*}P < 0.05$).

4. Discussion

We pretreated cultured human keratinocytes with various concentrations of monoclonal (mouse) and polyclonal (goat) anti IL-6 antibodies followed by stimulation with HD (10^{-4} M for 24 h at 37 °C) to evaluate the neutralization effects of these anti IL-6 antibodies. We tested for neutralization using ELISA and Luminex$^{100\,TM}$, and evaluated the expression of the corresponding gene, hIL-6, using RT-PCR.

Our findings can be summarized as follows: hIL-6 is present in significant amounts in human keratinocytes stimulated with HD; HD stimulation increased the expression of hIL-6 cytokine; HD induced expression of hIL-6 was due to gene activation; the hIL-6 levels in the HD-stimulated NHEK under the influence of anti IL-6 antibodies significantly decreased the levels as well as completely neutralized the hIL-6 in the medium.

Human IL-6 is a potent inflammatory cytokine produced by a variety of cells including epidermal and dermal fibroblasts (Kelso, 1998). Interleukin-6 (IL-6) is synthesized and released by normal epidermis and keratinocyte cells (Taga and Kuhimoto, 1990; Heinrich et al., 1990; Van Snick, 1990; Klein et al., 1991; Akira et al., 1993). Under normal conditions, hIL-6 secretion by keratinocytes appears weak to moderate. However, when stimulated with sulfur mustard keratinocytes can synthesize hIL-6. An earlier study demonstrated hIL-6 secretion in response to HD (Arroyo et al., 2001).

Another interesting finding from our study is that there are insignificant amounts of hIL-6 in human keratinocytes under basal conditions, contrasting with significant HD-induced hIL-6 expression due to increased transcription of hIL-6 gene. The mechanism of this effect remains to be determined.

Neutralization effects were observed (Table 3, Figs. 1–3) using IL-6 neutralizing antibodies in NHEK stimulated by HD. The secretion of hIL-6 induced by HD in NHEK was reduced with a monoclonal (mouse) anti IL-6 antibody by 76% (Table 3). The effect of monoclonal (mouse) anti IL-6 antibody seemed to have inhibitory effects on hIL-6 levels in the HD-stimulated keratinocytes. These effects

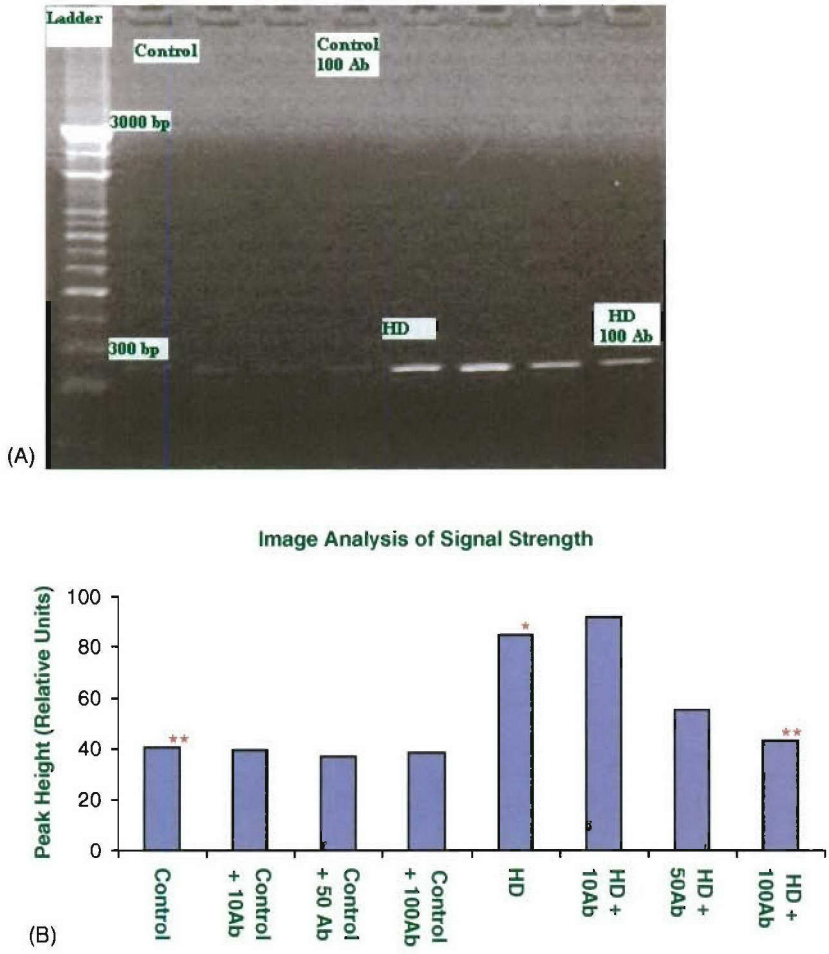

Fig. 3. (A) Differential expression of hIL-6 mRNA on pretreated NHEK with monoclonal anti IL-6 antibody and after stimulation with HD (10^{-4} M) for an additional 24 h. NHEK were treated with 10, 50, 100 μg/mL of monoclonal (mouse) anti IL-6 antibody (left side). PCR reactions were performed with a mixture of cDNA reverse transcripted from cellular RNA. Amplicons were resolved on 2% agarose gels and stained with ethidium bromide. Experiments were repeated five times. Increased expression of hIL-6 in HD-stimulated NHEK is shown in the right center. Decreased expression of HD-induced hIL-6 in NHEK treated with anti IL-6 antibody for 24 h followed HD-stimulation for additional 24 h (right side). RNA extraction and RT-PCR reactions were performed as described in Section 2. (B) Signal strength of the observed bands (~260 base pairs) reported as peak height in relative units.

were significant compared with polyclonal-goat anti IL-6 antibody.

Overall, HD exposure affects the synthesis of the inflammatory cytokine hIL-6 by activating the corresponding gene. Nevertheless, HD-induced cytokine production is relevant to the pathogenesis of HD injury. Blister formation is an undesired effect resulting from sulfur mustard exposure. Human IL-6 may contribute to the vesicant damage of the dermis (Arroyo et al., 1999, 2001, 2003). In view of the potential role of hIL-6 in the pathogenesis of human vesication, specific inhibitors of IL-6 might have therapeutic value. Our findings show that anti IL-6 antibodies, which may significantly reduce the observed inflammatory process, abrogate IL-6 induction by HD in human keratinocytes. The use of receptor antagonists and antibodies or receptors specific for inflammatory mediators such as IL-6 may provide the data to determine the role of inflammation in HD pathology and produce medical countermeasures for HD toxicity. Furthermore, the application of neutralizing monoclonal IL-6 antibody in patients with myeloma, HIV, and rheumatoid arthritis has shown promising results (Beck et al., 1994; de Hon et al., 1995; Sawamura et al., 1998).

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